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Original Article

Lack of promoting effect of titanium dioxide particles on chemically-induced skin carcinogenesis in rats and mice

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ABSTRACT — Nano-sized titanium dioxide particles (TiO_2) are widely used in cosmetics, sunscreens and food additives. We previously reported that topical application of non-coated rutile type TiO_2 did not exhibit a promoting effect on ultraviolet B-initiated skin carcinogenesis in rats, and that this was likely due to lack of penetration of TiO_2 into the epidermis. In the present study, we examined the promoting effect of silicone coated TiO_2 ($sTiO_2$) suspended in silicone oil and non-coated TiO_2 ($ncTiO_2$) suspended in Pentalan 408 on a two-stage skin chemical carcinogenesis model: $sTiO_2$ suspended in silicon oil forms smaller particles than $ncTiO_2$ suspended in Pentalan because of the smaller sizes of aggregates formed. The model used skin carcinogenesis-sensitive human c-Ha-ras proto-oncogene transgenic mice (rasH2) and rats (Hras128) and their wild-type counterparts and CD-1 mice to test the effects of topical application of TiO_2. Animals were initially treated with a single dose of 7,12-dimethylbenz[a]anthracene (DMBA) and then with 0, 10, or 20 mg $sTiO_2$ (mice) or 0, 50, or 100 mg $ncTiO_2$ (rats). The incidence and multiplicity of skin tumors (squamous cell papilloma and carcinoma) did not increase over DMBA alone controls in skin carcinogenesis-sensitive mice or rats or wild-type animals. Analysis of rat skin indicated that $sTiO_2$ and $ncTiO_2$ did not penetrate though either healthy or damaged skin. Furthermore $sTiO_2$ did not penetrate an *in vitro* human epidermis model. Our results indicate that treatment with $sTiO_2$ or $ncTiO_2$ did not promote skin carcinogenesis in mice or rats, probably due to lack of penetration through the epidermis.

Key words: Nano-size TiO_2, Skin carcinogenesis, Hras, Rat, Mouse

INTRODUCTION

Nano-sized titanium dioxide (TiO_2) particles are used in sunscreen formulations to protect against skin lesions caused by exposure to UV light (Gelis *et al.*, 2003; Rouabhia *et al.*, 2002; Suzuki, 1987). Nano and larger scale titanium dioxide particles are known to be carcinogenic to the rat lung (Baan *et al.*, 2006; Baan, 2007). Recently, we demonstrated a promoting effect on rat lung carcinogenesis by nano-size TiO_2 particles administered by a novel intrapulmonary spraying method (Xu *et al.*, 2010). The mechanism of promotion of lung carcinogenesis involved the induction of MIP1α protein expression by $ncTiO_2$-laden alveolar macrophages (Xu *et al.*, 2010).

We also examined the carcinogenic effect of TiO_2 (mean manufacturer's particulate diameter of 20 nm) on the skin in a UVB-initiated two-stage rat carcinogenesis model and found that topical application of TiO_2 did not promote skin carcinogenesis in this model. This result is probably due to the inability of TiO_2 to penetrate through

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the epidermis and reach the underlying tissue (Xu *et al.*, 2011). This speculation is consistent with a report by Newman *et al.* (2009) demonstrating an absence of penetration of TiO_2 through the epidermis and hair follicles (Newman *et al.*, 2009). On the other hand, Wu *et al.* (2009) reported that TiO_2 (4 nm and 60 nm length) could penetrate through the stratum corneum (SC) and become located in the deep layer of the epidermis after being topically applied to pig ear for 30 days (Wu *et al.*, 2009). These inconsistent observations may be due to differences in particle size and the animals used.

The skin is histologically composed of the SC, epidermis, dermis and the subcutaneous tissue. The SC is the rate-limiting barrier against exposure to various exogenous chemical and physical agents (Schaefer *et al.*, 2003). For solid materials, including nano-sized particles, to cause inflammatory lesions, they need to penetrate the SC to interact with macrophages and other inflammatory leucocytes. Long-term activation of inflammatory leukocytes has the potential to cause skin carcinogenesis. Thus, the potential skin-carcinogenicity of TiO_2 is dependent on its size and ability to penetrate through the SC.

The surface of the TiO_2 used in cosmetics is usually coated with aluminum oxide or silicone oils to prevent aggregate formation and to enhance dispersal (Nohynek *et al.*, 2008). The particle size of TiO_2 suspended in silicone oils is known to be smaller than that of non-coated TiO_2 suspensions (Senzui *et al.*, 2010; also compare Fig. 1E with Fig. 1F). In our previous study, we showed that rutile type non-coated TiO_2 ($ncTiO_2$) did not penetrate the epidermal tissue and thus did not cause promotion of chemically-induced skin carcinogenesis. In the present study, we used rutile type TiO_2 coated with silicone ($sTiO_2$) suspended in silicon oil to minimize aggregation and improve the penetrating ability of the particles.

The ability of $sTiO_2$ suspended in silicone oil and $ncTiO_2$ suspended in Pentalan408 to promote skin carcinogenesis was examined using the 7,12-dimethylbenz[a] anthracene (DMBA)-initiated skin carcinogenesis model employing skin carcinogenesis-sensitive animals and their wild-type counterparts as the test animals. The rasH2 mouse carries a human c-Ha-ras proto-oncogene and is highly susceptible to chemically induced skin carcinogenesis (Muto *et al.*, 2006). The Hras128 rat also carries a human c-Ha-ras proto-oncogene and highly susceptible to chemically induced skin carcinogenesis (Park *et al.*, 2004).

In addition to the animal models, we also used an *in vitro* model to examine $sTiO_2$ particle penetration into skin. Unlike animal skin, the *in vitro* model does not have hair follicles, allowing direct examination of the ability of $sTiO_2$ particles to penetrate through a layer of human skin epidermal keratinocytes.

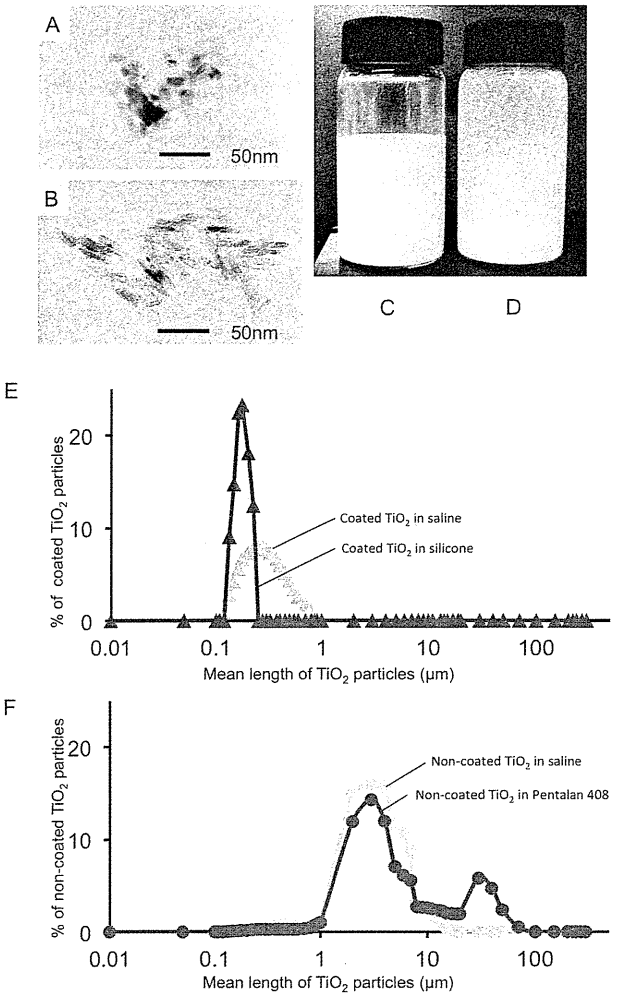

Fig. 1. Physicochemical features of $sTiO_2$ / $ncTiO_2$. $sTiO_2$ particles were round to oval in shape (A), and $ncTiO_2$ particles were club shaped (B). $sTiO_2$ particles remained evenly dispersed in silicone solution (C) while $ncTiO_2$ particles formed a white sediment at the bottom of the bottle 3 days after preparation (D). Size distribution of $sTiO_2$ suspended in saline (gray triangles) and in silicone (black triangles) (E). Size distribution of $ncTiO_2$ suspended in saline (gray circles) and in Pentalan 408 (black circles) (F). % of TiO_2 particles (Y axis) was calculated as the ratio of TiO_2 particles of a particular mean length/total particles examined.

Table 1. TiO_2 materials and animal strains used in this study

Coating status of TiO_2	Size	Concentrartion of TiO_2 (mg/ml)	Suspended in	Skin assay (Carcinogen) Strain (or *in vitro* system)
Coated (rutile type)	35 nm	50, 100	Silicone	Carcinogenesis (DMBA) rasH2 mouse, C57BL mouse
		100, 200	Silicone	Penetration LabCyte EPI-MODEL
Non-coated (rutile type)	20 nm	100, 200	Pentalan 408	Carcinogenesis (DMBA) Hras128 rat, Sprague-Dawley rat
		50, 100	Pentalan 408	Carcinogenesis (DMBA) CD1 mouse
		200	Pentalan 408	Penetration Sprague-Dawley rat

MATERIALS AND METHODS

Animals

Male rasH2 mice and Hras128 rats, known to be highly sensitive to chemically induced skin carcinogenesis (Muto *et al.*, 2006; Park *et al.*, 2004), and their wild-type counterparts, CB6F1 mice and SD rats, were purchased from CLEA Japan Co., Ltd. (Tokyo, Japan). To confirm the results, CD-1 mice, which are frequently used in skin carcinogenesis studies, were also included in this series of studies. The animals were housed in the animal center of Nagoya City University Medical School, maintained on a 12 hr light-dark cycle and received Oriental MF basal diet (Oriental Yeast Co., Tokyo, Japan) and water *ad libitum*. All animals were kept for 1 week for acclimation. The experiments were conducted according to the Guidelines for the Care and Use of Laboratory Animals, and the study protocol was approved by the Institutional Animal Care and Use Committee of Nagoya City University Medical School.

Preparation of suspensions of titanium dioxide (TiO_2) and size analysis

$sTiO_2$ particles (silicone coated, mean manufacturer's particulate diameter of 35 nm) and $ncTiO_2$ particles (rutile type, mean manufacturer's particulate diameter of 20 nm) were provided by Japan Cosmetic Association, Tokyo, Japan. Size, coating, dose and suspension vehicles and animals used are summarized in Table 1. The size distribution of $sTiO_2$ suspended in silicone oil (cyclopentasiloxane, KF-995, Shin-Etsu Chemicals Co., Tokyo, Japan) or in saline and $ncTiO_2$ particles suspended in Pentalan 408 (pentaerythritol tetraethylhexanoate, CAS7299-99-2, Nikko Chemicals Co., Tokyo, Japan) or in saline was determined by a Particle Size Distribution Analyzer (Shimadzu Techno-Research Inc., Kyoto, Japan). The shape of suspended $sTiO_2$ and $ncTiO_2$ was observed by transmission electron microscopy (JEOL Co. Ltd., Tokyo, Japan). Freshly made suspensions were sonicated for 30 min, and suspensions were sonicated again for 30 min just prior to use.

Experimental design

Skin carcinogenesis study of silicone coated TiO_2 ($sTiO_2$) using rasH2 mice

The back skin of 7-week-old female rasH2 mice (60 mice) and wild-type CB6F1 mice (60 mice) was shaved (2 × 2 cm area) and the animals received a single topical application (painting) of 0.1 ml DMBA solution (2 mg/ml in acetone). Two weeks later, the animals were divided into 3 groups and the area which was painted with DMBA was shaved and painted with silicon oil alone or $sTiO_2$ suspended in silicon oil 5 times a week until termination of the experiment: Group 1 mice (15 mice of each strain) were painted with 0.2 ml silicone oil; group 2 mice (15 mice of each strain) were painted with 0.2 ml of 50 mg/ml $sTiO_2$ suspended in silicone oil; group 3 mice (15 mice of each strain) were painted with 0.2 ml of 100 mg/ml $sTiO_2$ suspended in silicone oil. Group 4 consisted of 15 mice of each strain painted with 0.2 ml 100 mg/ml $sTiO_2$ suspended in silicon oil 5 times a week without prior DMBA treatment. The rasH2 mice were killed at experimental week 8 and wild-type CB6F1 mice were killed at experimental week 40.

Skin carcinogenesis study of non-coated TiO_2 ($ncTiO_2$) using Hras128 rats

The back skin of 10-week-old male Hras128 rats (50 rats) and wild-type SD rats (36 rats) was shaved (3 × 3 cm area) and the animals received a single topical application (painting) of 0.5 ml DMBA solution (5 mg/ml in acetone)

(Park *et al.*, 2004). Two weeks later, the animals were divided into 3 groups and the area which was painted with DMBA was shaved and painted with Pentalan 408 alone or $ncTiO_2$ suspended in Pentalan 408 twice a week until termination of the experiment: Group 1 rats (17 Hras128 and 12 SD rats) were painted with 0.5 ml Pentalan 408 alone; group 2 rats (16 Hras128 and 12 SD rats) were painted with 0.5 ml of 100 mg/ml $ncTiO_2$ suspended in Pentalan 408; group 3 rats (17 Hras128 and 12 SD rats) were painted with 0.5 ml of 200 mg/ml $sTiO_2$ suspended in Pentalan 408. The Hras128 rats were killed at experimental week 28 and wild-type SD rats were killed at experimental week 40.

Skin carcinogenesis study of non-coated TiO_2 ($ncTiO_2$) using wild-type CD1 mice

The back skin of 10-week-old female CD1 mice (62 mice) was shaved (2 × 2 cm area) and the animals received a single topical application (painting) of 0.1 ml DMBA solution (2 mg/ml in acetone). Two weeks later, the animals were divided into 4 groups and the area which was painted with DMBA was shaved and painted with Pentalan 408 alone twice a week, $ncTiO_2$ suspended in Pentalan 408 twice a week, or TPA 4 times a week (positive control) until termination of the experiment: Group 1 mice (16 mice) were painted with 0.2 ml Pentalan 408; group 2 mice (16 mice) were painted with 0.2 ml of 50 mg/ml $ncTiO_2$ suspended in Pentalan 408; group 3 mice (15 mice) were painted with 0.2 ml of 100 mg/ml $ncTiO_2$ suspended Pentalan 408; group 4 mice (15 mice) were painted with 0.2 ml TPA solution (200 nmol/ml in acetone). Group 1-3 mice were killed at experimental week 52; group 4 mice were killed at experimental week 40.

Skin penetration study of non-coated TiO_2 ($ncTiO_2$) in SD rats

Based on our previous study showing lack of TiO_2 penetration through the normal skin (Xu *et al.*, 2011), $ncTiO_2$ was applied to damaged skin, which is postulated to be more susceptible to particle penetration. The back skin of 10-week-old female SD rats (24 rats) was shaved (3 × 3 cm area) and the epidermis was removed by stripping the epidermis off with a fresh piece of adhesive tape (3M's No. 3760, Scotch Mending Tape, Sumitomo 3M Ltd., Tokyo, Japan): Stripping was done 30 times to completely remove the epidermis. The epidermis-stripped skin was then painted with 0.5 ml of Pentalan or 0.5 ml of 200 mg/ml $ncTiO_2$ suspended in Pentalan 408 at 4-day-intervals over the course of 3 and a half weeks (7 treatments in 3½ weeks). Localization of $ncTiO_2$ particles in the epidermis was determined by histological observation. Skin tissue samples were taken at 1, 3, and 7 days after stripping to examine recovery of the epidermis and penetration of $ncTiO_2$ into the skin.

Skin penetration study of silicone coated TiO_2 ($sTiO_2$) in the in vitro skin model

To evaluate whether optimally dispersed $sTiO_2$ particles could penetrate into the epidermis, we applied $sTiO_2$ particles dispersed in silicone oil to the LabCyte EPI-MODEL kit (Japan Tissue Engineering Co. Ltd., Aichi, Japan), which is constructed of human skin epidermis keratinocytes on a mesh over a receiving chamber. In 12 wells: 4 wells had silicone oil alone applied directly to the human skin epidermis keratinocytes for 48 hr; 4 wells had 100 mg/ml $sTiO_2$ suspended in silicone oil applied directly to the human skin epidermis keratinocytes for 48 hr; and 4 wells had 200 mg/ml $sTiO_2$ suspended in silicone oil applied directly to the human skin epidermis keratinocytes for 48 hr. The medium in the receiving chamber was collected for elemental titanium analysis by an inductively coupled plasma/mass spectrometry (ICP-MS) (HP-4500, Hewlett-Packard Co., Houston,TX, USA) as described previously (Xu *et al.*, 2011).

Statistical analysis

Statistical analysis was performed using the Kruskal-Wallis and Bonferroni-Dunn's multiple comparison tests. Statistical significance was analyzed using a two-tailed Student's *t*-test and Bonferroni-Dunn's multiple comparison test. A value of $P < 0.05$ was considered to be significant.

RESULTS

Size distribution of $ncTiO_2$ and $sTiO_2$ particles

Transmission electron microscopy (TEM) analysis showed that the shape of $sTiO_2$ particles was generally round to oval (Fig. 1A), while $ncTiO_2$ particles were more clubbed shaped (Fig. 1B). The $sTiO_2$ in silicone oil solutions remained without obvious sedimentation for 3 days after preparation (Fig. 1C). In contrast, the $ncTiO_2$ in Pentalan 408 solutions contained considerable sedimentation 3 days after preparation (Fig. 1D). The particle size distribution of $sTiO_2$ and $ncTiO_2$ solutions is shown in Figs. 1E and 1F. The mean length of $sTiO_2$ particles suspended in saline and silicone was 0.16 ± 0.07 and 0.28 ± 0.22 μm, respectively (Fig. 1E). The mean length of $ncTiO_2$ particles suspended in saline and Pentalan 408 was 3.18 ± 0.35 and 4.97 ± 0.50 μm, respectively (Fig. 1F). These results indicate that $sTiO_2$ in silicone oil remained dispersed for a longer time than $ncTiO_2$ in Pentalan 408.

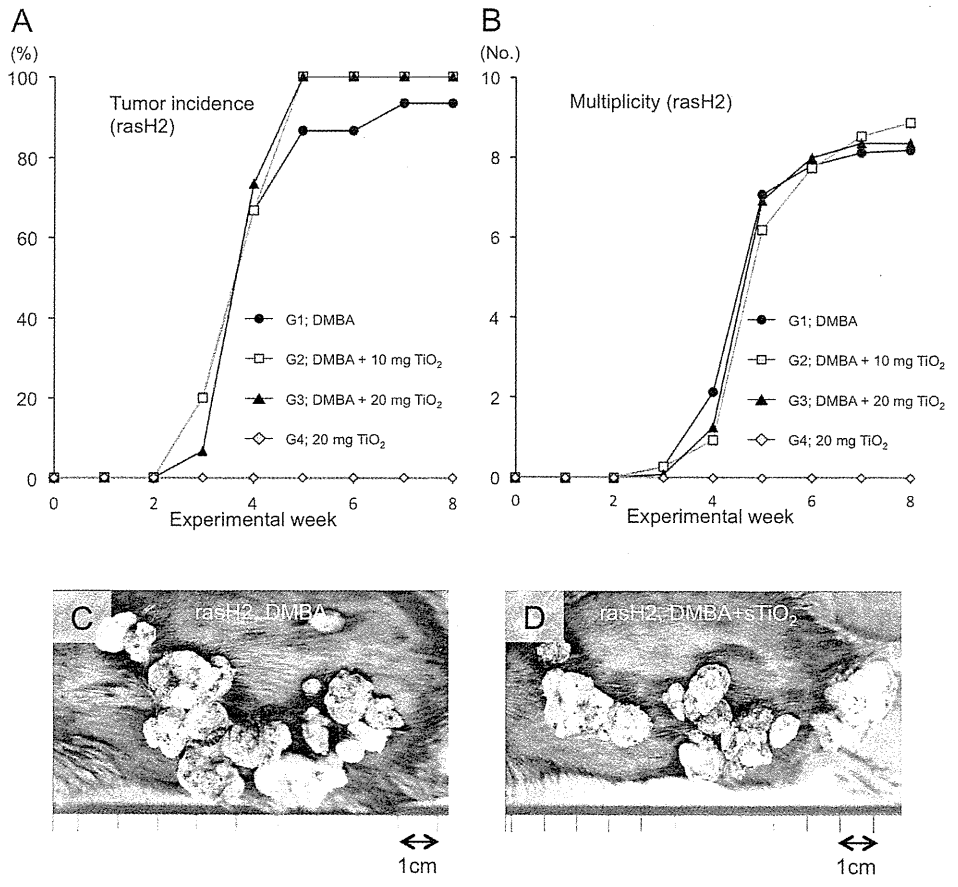

Fig. 2. Effects of $sTiO_2$ in a two-stage carcinogenesis model using rasH2 mice. The incidence of skin tumors in rasH2 mice: DMBA treated group (black circles), DMBA followed by treatment with 10 mg $sTiO_2$ (gray squares) or 20 mg $sTiO_2$ (black triangles). 20 mg $sTiO_2$ treated group (gray diamonds) (A). Multiplicity of skin tumors in rasH2 mice: DMBA treated group (black circles), DMBA followed by treatment with 10 mg $sTiO_2$ (gray squares) or 20 mg $sTiO_2$ (black triangles). 20 mg $sTiO_2$ treated group (gray diamonds) (B). Representative macroscopic appearance of skin tumors initiated with DMBA in rasH2 mice (C). The gross morphology of the tumors did not show obvious differences between the DMBA (C) and DMBA + $sTiO_2$ (D) groups.

Skin carcinogenesis study of $sTiO_2$ in silicone oil using rasH2 mice

Figures 2A and 2B show the time lapse incidence and multiplicity (number/mouse) of macroscopic skin tumors in rasH2 mice. No statistically significant differences were found between $sTiO_2$ treated rasH2 mice (groups 2 and 3: DMBA plus 50 mg/ml and 100 mg/ml $sTiO_2$, respectively) and control rasH2 mice (group 1: DMBA alone) (Figs. 2A and 2B, Table 2-1). Similarly, no statistically significant differences were found between treated and control groups of wild-type CB6F1 mice (Table 2-2). No tumors were induced in group 4 ($sTiO_2$ alone) of either rasH2 (Figs. 2A and 2B, Table 2-1) or wild-type CB6F1 mice (Table 2-2). Skin tumors were histologically SCP and SCC in rasH2 and wild-type CB6F1 mice. Representative macroscopic skin tumors induced by DMBA alone and by DMBA plus $sTiO_2$ are shown in Fig. 2C and 2D, respectively.

Skin carcinogenesis study of $ncTiO_2$ using Hras128 rat

Figures 3A and 3B show the time lapse incidence and multiplicity (number/rat) of macroscopic skin tumors in Hras128 rats. No statistically significant differences were found between $sTiO_2$ treated and control rasH2 rats (Figs. 3A and 3B, Table 3-1). Similarly, no statistically significant differences were found between treated and control groups of wild-type SD rats (Table 3-2).

Microscopically, TiO_2 was not observed within the SCP or SCC tissue (Figs. 4A and 4B). TiO_2 was observed

Table 2-1. Effects of $sTiO_2$ on skin carcinogenesis in rasH2 mice

Group	Treatment	No. of mice	SCP		SCC		SCP + SCC	
			Incidence (%)	Multiplicity	Incidence (%)	Multiplicity	Incidence (%)	Multiplicity
1	DMBA + Silicone	15	14 (93)	7.27 ± 4.74	5 (33)	0.60 ± 0.99	14 (93)	7.87 ± 5.17
2	DMBA + 10 mg TiO_2	15	15 (100)	8.13 ± 3.66	9 (60)	1.00 ± 1.00	15 (100)	9.13 ± 3.76
3	DMBA + 20 mg TiO_2	15	15 (100)	6.80 ± 3.88	8 (53)	0.73 ± 0.80	15 (100)	7.53 ± 3.31
4	20 mg TiO_2	15	0	0	0	0	0	0

SCP, squamous cell papilloma; SCC, squamous cell carcinoma.
Multiplicity: number of tumors per mouse.

Table 2-2. Effects of $sTiO_2$ on skin carcinogenesis in wild-type CB6F1 mice

Group	Treatment	No. of mice	SCP		SCC		SCP + SCC	
			Incidence (%)	Multiplicity	Incidence (%)	Multiplicity	Incidence (%)	Multiplicity
1	DMBA + Silicone	15	1 (7)	0.07 ± 0.26	1 (7)	0.07 ± 0.26	2 (13)	0.13 ± 0.35
2	DMBA + 10 mg TiO_2	15	2 (13)	0.13 ± 0.35	0	0	2 (13)	0.13 ± 0.35
3	DMBA + 20 mg TiO_2	15	2 (13)	0.20 ± 0.56	0	0	2 (13)	0.20 ± 0.56
4	20 mg TiO_2	15	0	0	0	0	0	0

SCP, squamous cell papilloma; SCC, squamous cell carcinoma.
Multiplicity: number of tumors per mouse.

on the surface and in the upper SC tissue and upper part of the hair follicles, but not in the underlying epidermis, dermis or subcutaneous tissues (Figs. 4C and 4D).

Skin carcinogenesis study of $ncTiO_2$ using wild-type CD1 mice

No statistically significant differences in tumor incidence or multiplicity was observed between treated and control groups of CD1 mice (Table 4). TPA treatment after DMBA significantly increased the incidence and multiplicity of SCP ($P < 0.001$).

Skin penetration study of $ncTiO_2$ in SD rats

Figures 5 A-D shows skin tissue samples collected before (Fig. 5A) and 1, 3 and 7 days after (Figs. 5B-D) removing the epidermis by tape-stripping. On day 1 the epidermis was completely removed (Fig. 5B), and a mass of fibrin exudate and underlying granulation tissue rich with neutrophils was the main feature of the skin surface. On day 3, regenerated epidermis already covered the granulation tissue (Fig. 5C). On day 7, the surface of the skin was fully covered by regenerated keratinocytes showing cornification and had an almost normal appearance (Fig. 5D).

The shaved back skin of SD-rats was painted with 100 mg TiO_2 suspended in Pentalan 408. Fig. 5E shows the presence of $ncTiO_2$ particles in the SC layer of the skin of these animals. Extensive histological observation failed to detect TiO_2 within the epidermis or dermis. In another series of experiments, the epidermis was removed from the back skin of SD rats by tape-stripping and the freshly stripped skin was painted with 100 mg $ncTiO_2$ suspended in Pentalan 408. This was repeated every 4 days for 3½ weeks (7 times total). Figure 5F shows the presence of TiO_2 on the surface of the regenerating epidermis 1 day after the last stripping/painting procedure. Extensive

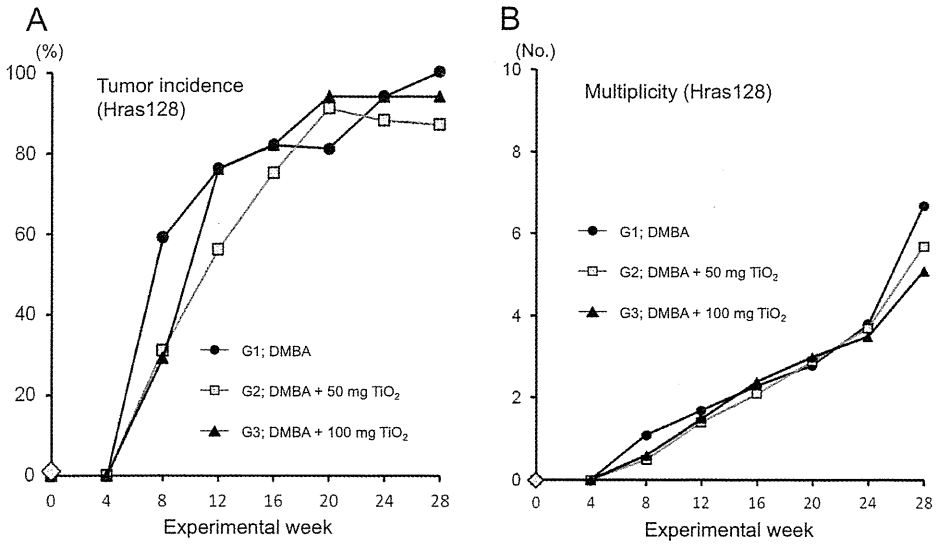

Fig. 3. Effects of $ncTiO_2$ in a two-stage carcinogenesis model using Hras128 rats. The incidence of skin tumors in Hras128 rats: DMBA treated group (black circles), DMBA followed by treatment with 50 mg $ncTiO_2$ (gray squares) or 100 mg $ncTiO_2$ (black triangles) (A). Multiplicity of skin tumors in rasH2 mice: DMBA treated group (black circles), DMBA followed by treatment with 100 mg $ncTiO_2$ (gray squares) or 200 mg $ncTiO_2$ (black triangles) (B).

Table 3-1. Effects of $ncTiO_2$ on skin carcinogenesis in Hras128 rats

Group	Treatment	No. of rats	SCP		SCC		SCP + SCC	
			Incidence (%)	Multiplicity	Incidence (%)	Multiplicity	Incidence (%)	Multiplicity
1	DMBA + Pentalan 408	17	16 (94)	9.65 ± 7.05	0	0	16 (94)	9.65 ± 7.05
2	DMBA + 50 mg TiO_2	16	14 (88)	6.81 ± 6.21	2 (13)	0.19 ± 0.54	14 (88)	7.00 ± 6.52
3	DMBA + 100 mg TiO_2	17	16 (94)	7.59 ± 3.86	2 (12)	0.12 ± 0.331	16 (94)	7.71 ± 3.93

SCP, squamous cell papilloma; SCC, squamous cell carcinoma.
Multiplicity: number of tumors per rat.

Table 3-2. Effects of $ncTiO_2$ on skin carcinogenesis in wild-type SD rats

Group	Treatment	No. of rats	SCP		SCC		SCP + SCC	
			Incidence (%)	Multiplicity	Incidence (%)	Multiplicity	Incidence (%)	Multiplicity
1	DMBA + Pentalan 408	12	3 (25)	0.25 ± 0.45	0	0	3 (25)	0.25 ± 0.45
2	DMBA + 50 mg TiO_2	12	2 (17)	0.17 ± 0.39	2 (17)	0.17 ± 0.39	4 (33)	0.33 ± 0.49
3	DMBA + 100 mg TiO_2	12	1 (8)	0.08 ± 0.29	0	0	1 (8)	0.08 ± 0.29

SCP, squamous cell papilloma; SCC, squamous cell carcinoma.
Multiplicity: number of tumors per rat.

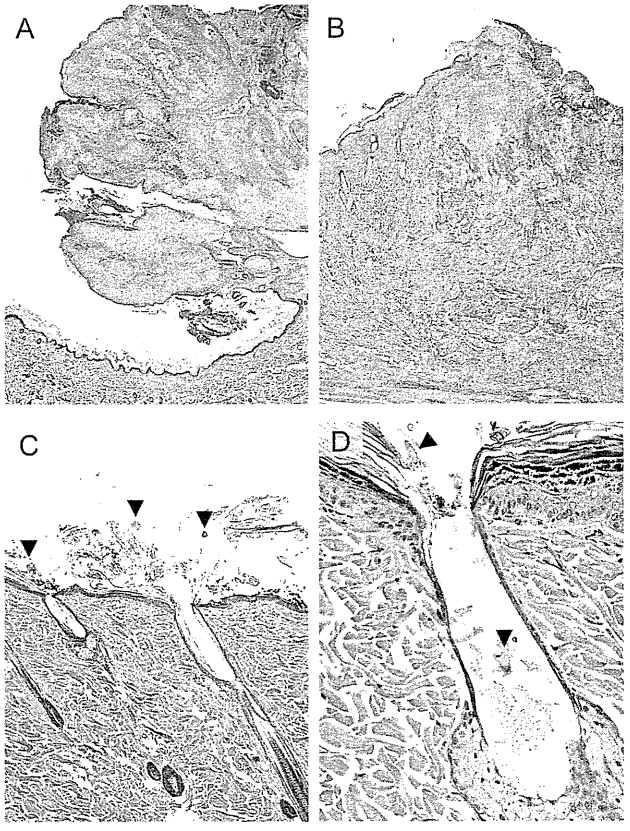

Fig. 4. Effects of $ncTiO_2$ in a two-stage carcinogenesis model. Representative histological features of SCP (A) and SCC (B) on the back skin of a Hras128 rat. TiO_2 aggregates were seen on the surface of stratum corneum (C, arrowheads), but not in the underlying epidermis, dermis or subcutaneous tissue. Some aggregates were found in the upper layer of stratum corneum (arrowheads) and in the lumen of the hair follicle (D) but not in the dermis.

histological observation failed to detect any TiO_2 particles in the underlying epidermis or dermis, indicating that $ncTiO_2$ failed to penetrate into the stripped skin. In addition, no inflammatory lesions were found in the epidermis or subcutaneous tissue of these animals. These results indicate that TiO_2 particles do not penetrate into the epidermis of either normal or damaged skin.

In vitro skin penetration study

The amount of elemental titanium in the receiving chamber did not show any significant increase over the vehicle group (Table 5). All the observed values are equivalent to background. The results indicate that $sTiO_2$ particles did not penetrate the human epidermis in this model.

DISCUSSION

TiO_2 particles, including both nano and larger sized particles, are known to be carcinogenic to the rat lung (Baan *et al.*, 2006). We have shown that alveolar macrophages play an important role in promotion of lung carcinogenesis when TiO_2 particles are inhaled into the lung (Xu *et al.*, 2011). Because of this, TiO_2 particles, especially nano-sized particles, are deemed to have the potential to induce skin tumors after long-term topical application should the particles penetrate into the epidermis and subcutaneous tissue and interact with macrophages. The current study is the first systematic study of the skin promotion/carcinogenesis effects of TiO_2 in a two-stage chemical carcinogenesis animal model. We found that even the smallest available sized TiO_2 ($sTiO_2$) did not exhibit promoting effects on the highly sensitive rasH2 mouse skin carcinogenesis model. Furthermore, we observed that TiO_2 without coating ($ncTiO_2$) did not cause skin tumor promotion in the skin carcinogenesis-sensitive Hras128 rat model or in the CD1 mouse. These results are in agreement with another recent study reporting the lack of carcinogenicity of topically applied TiO_2 (Furukawa *et al.*, 2011).

We also found that topically applied $ncTiO_2$ did not penetrate normal rat skin or skin which had the epidermis completely removed, nor did $sTiO_2$ penetrate the *in vitro* human epidermis model. Thus, the lack of skin promotion/carcinogenesis effects is probably due to lack of penetration of the particles through the epidermis to the dermis where cytogenetic cells of skin carcinogenesis reside. In another study, we found no promoting effect of TiO_2 particles in a UVB-initiated long-term (52 weeks) skin carcinogenesis study and no penetration of TiO_2 particles through the epidermis (Xu *et al.*, 2011).

Results showing lack of TiO_2 penetration through the epidermis are in accordance with other reports. Numerous *in vitro* and *in vivo* studies using murine, porcine, or human skin have shown that nano-sized TiO_2 does not penetrate the skin (reviewed in Nohynek *et al.* (2008)). In addition to these studies, Gottbrath *et al.* (2003) report that after topical application of TiO_2 to the underside of the forearm of a human volunteer, ultrafine TiO_2 did not penetrate beyond the SC.

Contrary to these findings, there is a single report by Wu *et al.* (2009) that TiO_2 particles (4 and 60 nm) did penetrate into the deep layers of the epidermis after topical application to the pig ear for 30 days (Wu *et al.*, 2009). They also reported that nano-size TiO_2 particles could penetrate the skin of hairless mice after 60 days dermal exposure, although they did not examine promotion/car-

Table 4. Effects of $ncTiO_2$ on skin carcinogenesis in wild-type CD1 mice

Group	Treatment	No. of mice	SCP		SCC		SCP + SCC	
			Incidence (%)	Multiplicity	Incidence (%)	Multiplicity	Incidence (%)	Multiplicity
1	DMBA + Pentalan 408	16	3 (19)	0.25 ± 1.30	0	0	3 (19)	0.25 ± 0.58
2	DMBA + 10 mg TiO_2	16	1 (6)	0.06 ± 0.25	0	0	1 (6)	0.06 ± 0.25
3	DMBA + 20 mg TiO_2	15	2 (13)	0.13 ± 0.35	0	0	2 (13)	0.13 ± 0.35
4	DMBA + TPA	15	13 (87)*	2.00 ± 1.41*	2 (13)	0.13 ± 0.35	13 (87)*	2.00 ± 1.41*

* Significantly different from group 1 (control) by Student's t-test ($p < 0.001$).
SCP, squamous cell papilloma; SCC, squamous cell carcinoma.
Multiplicity: number of tumors per mouse.

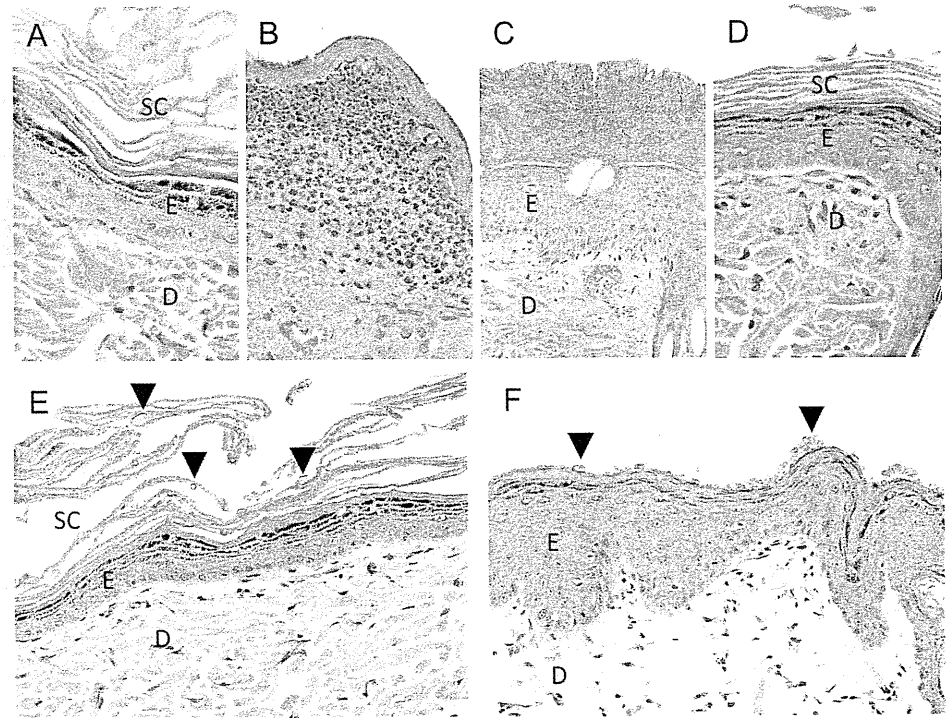

Fig. 5. Histological features of the skin after striping away the epidermis and study of $ncTiO_2$ penetration into normal and stripped skin using wild-type Sprague-Dawley rats. The back skin of SD rats was shaved and the epidermis was left intact or stripped away using tape. Stratum corneum (SC), epidermis (E), and dermis (D) are intact in the shaved, not stripped group (A). In the tape-stripped group, one day after stripping away the epidermis, the stripping site is covered with fibrin exudates with rich neutrophilic infiltration (B). Three days after stripping, regenerated epidermis (E) is present underneath the exudate (C). Seven days after stripping, the regenerated epidermis (E) is composed of mature keratinocytes and a stratum corneum (SC) (D). The back skin of SD rats was shaved (but the epidermis was not stripped away) and painted with $ncTiO_2$ suspended in Pentalan 408. $ncTiO_2$ aggregates are present in the stratum corneum (SC) (arrowheads, brown material) of the skin of these animals (E); particles were not detected within the underlying skin tissue. Freshly stripped skin was painted with $ncTiO_2$ suspended in Pentalan 408, and this process was repeated every 4 days over the course of $3^1/_2$ weeks (7 times total). $ncTiO_2$ particles (arrowheads, brown material) are present on the surface of the skin one day after the last stripping/painting procedure (F); particles were not detected in the underlying tissue.

Table 5. *In vitro* penetration of $sTiO_2$ particles

Treatment	Amount of elemental titanium in the receiving chamber (μg/ml)
Silicone oil	0.11 ± 0.01
100 mg/ml $sTiO_2$	0.14 ± 0.01
200 mg/ml $sTiO_2$	0.12 ± 0.01
None	0.11 ± 0.03

cinogenesis effects of the particles (Wu *et al.*, 2009). Differences in experimental systems (i.e., animal strain used in the experiment, exposure period, particle suspension, mean primary/actual length of the particle, and TiO_2 manufacturer) may possibly explain the discrepancies reported on TiO_2 particle penetration.

Two studies have reported finding TiO_2 particles in hair follicles (Bennat and Muller-Goymann, 2000; Lekki *et al.*, 2007). These studies taken together with Wu *et al.* (2009) suggest the possibility that the hair follicle may be a route of skin penetration by TiO_2 particles. However, we found that TiO_2 particles remained primarily in the SC and the upper lumen of hair follicles. Bennat and Muller-Goymann (2000) and Lekki *et al.* (2007) also report that while topically applied TiO_2 was found in hair follicules, it did not penetrate into the underlying tissue or sebaceous glands.

Penetration of TiO_2 into underlying dermal tissues even after removing the entire epidermis by tape-stripping did not occur. Rather, aggregates of TiO_2 particles were found on the exterior of the SC exhibiting regeneration and no particles were found in the underlying tissues. The freshly manufactured TiO_2 particles used in the present study primarily measured 20-35 nm in their longer diameter. These particles are lyophobic and easily form micro-sized aggregates (160-5,000 nm in length), and these larger particles are unable to penetrate through the epidermis.

In summary, nano-sized TiO_2 particles, even silicone coated TiO_2 suspended in silicone oil which provides optimal dispersion of the particles, did not penetrate the epidermis of rat or human skin models and did not exhibit promoting effects in rat or mouse two-stage skin carcinogenesis models. Therefore, topical application of TiO_2 was not carcinogenic, and this lack of carcinogenicity is likely due to the lack of penetration through the epidermis. Our studies taken together with other reports lead us to conclude that topical application of TiO_2 to human skin is very unlikely to pose a potential risk to human health.

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